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Gamete Fusion Assay in Mice

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[Abstract] Gamete fusion, which is the final event of fertilization, is a crucial physiological event in the creation of a new fetus. In mammals, sperm IZUMO1 and oocyte IZUMO1R (JUNO) recognition play a role in triggering this process. Gamete fusion occurs through a complex but steady and unfailing intermolecular reaction because fertilization must ensure species specificity, in which fusion takes place between gametes of the same species only. Although many factors involved in this process have recently been identified, their specific contributions remain largely unknown. The current article describes detailed methods for assessment of gamete fusion in mice, visualized by fluorescent dye transfer, from unfertilized oocyte to spermatozoa. These methods are applicable not only for fixed cells but also live imaging of gametes.

Keywords: Spermatozoon, Oocyte, Fertilization, Gamete fusion, Mouse

[Background] Mammalian fertilization is completed by cell-cell fusion, which is one of the most common physiological events. There are many obstacles that hinder the sperm's capability to reach the oocyte. Among the enormous number of ejaculated spermatozoa (approximately 300 million in humans), only one spermatozoon is admitted to fertilize one oocyte.

Gamete recognition and fusion in mammals are part of a strict selection process in the molecular machinery of fertilization because gametes among different species are usually unable to merge with each other. These processes are likely carried out through a complex intermolecular interaction, in which izumo sperm-egg fusion 1 (IZUMO1) (Inoue *et al.*, 2005), sperm acrosome associated 6 (SPACA6) (Lorenzetti *et al.*, 2014; Barbaux *et al.*, 2020; Noda *et al.*, 2020), transmembrane protein 95 (TMEM95) (Lamas-Toranzo *et al.*, 2020; Noda *et al.*, 2020), fertilization influencing membrane protein (FIMP) (Fujihara *et al.*, 2020), sperm-oocyte fusion required 1 (SOF1) (Noda *et al.*, 2020), the newly-identified dendrocyte expressed seven transmembrane protein (DC-STAMP) domain-containing 1 (DCST1) and its paralogue DCST2 (Inoue *et al.*, 2021) on the sperm side, and JUNO (also known as IZUMO1 receptor) (Bianchi *et al.*, 2014) and cluster of differentiation 9 (CD9) (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000) on the ovum side, all participate in gamete fusion, as proven by gene disruption. Among them, the IZUMO1-JUNO regulation system is likely essential for triggering gamete fusion (Inoue *et al.*, 2013 and 2015; Aydin *et al.*, 2016; Ohto *et al.*, 2016), although how these factors contribute to gamete fusion remains unclear (Bianchi and Wright, 2020).

Mainly three different methods can be used for preparing zona pellucida (ZP)-free oocytes. The Acidic Tyrode's solution method is a general method that has been used by many researchers because

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preparation is quick and easy. However, long exposure to Acidic Tyrode's solution causes the oocytes to die easily. Although in this study there were no obvious differences in the surface structure of Acidic Tyrode's solution- and collagenase-treated ZP-free oocytes (Figure 2A), a remnant of dissolved ZP was found adhered on the oocyte plasma membrane only when the Acidic Tyrode's solution method was used (Figure 2B), resulting in acrosome-intact spermatozoa binding to the surface of oocytes, which is considered a false binding (Yamagata *et al.*, 2002). These problems are resolved by collagenase and mechanical ZP removal methods (Yamagata *et al.*, 2002) (Figure 2B). However, regarding the mechanical ZP removal method, both expensive equipment and micromanipulator skills are required (Inoue and Okabe, 2008); therefore, the collagenase method is recommended as it is simple and does not require any specialized equipment or skill (Yamatoya *et al.*, 2011).

Materials and Reagents

1. 0.22-μm pore size polyethersulfone membrane (Merck, catalog number: S2GPU11RE)
2. 1-ml, 26-gauge, 1/2-inch syringe (TERUMO CORPORATION, model: SS-01T2613S)
3. 35-mm uncoated plastic dish plastic dish (IWAKI, model: 1000-035)
4. 60-mm uncoated plastic dish plastic dish (IWAKI, model: 1010-060)
5. ≥ 8-week-old female mice
6. ≥ 12-week-old male mice
7. Acidic Tyrode's solution (Merck, catalog number: T1788)
8. AlbuMAX™ I Lipid-Rich BSA (Thermo Fisher Scientific, catalog number: 11020021)
9. $CaCl_2 \cdot 2H_2O$ (Merck, catalog number: C7902)
10. Collagenase (FUJIFILM Wako Pure Chemical Corporation, catalog number: 038-22361)
11. Equine chorionic gonadotropin (eCG) (ASKA Animal Health)
12. FHM medium (Merck, catalog number: MR-024-D)
13. Glucose (Merck, catalog number: G6152)
14. Glutaraldehyde solution 20% (w/v) (FUJIFILM Wako Pure Chemical Corporation, catalog number: 072-02262)
15. Hoechst 33342 (Thermo Fisher Scientific, catalog number: H1399)
16. Human chorionic gonadotropin (hCG) (ASKA Animal Health)
17. Hyaluronidase type IV-S (Merck, catalog number: H4272)
18. KCl (Merck, catalog number: P5405)
19. KH_2PO_4 (Merck, catalog number: P5655)
20. $MgSO_4 \cdot 7H_2O$ (Merck, catalog number: 63138)
21. Mineral oil (Merck, catalog number: M8410)
22. NaCl (Merck, catalog number: S5886)
23. $NaHCO_3$ (Merck, catalog number: S5761)
24. Penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation, catalog number: 168-23191)

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25. Phenol red 0.04% (w/v) (FUJIFILM Wako Pure Chemical Corporation, catalog number: 163-20623)
26. Sodium pyruvate (Merck, catalog number: P4562)
27. Sterile water, endotoxin free (FUJIFILM Wako Pure Chemical Corporation, catalog number: 196-15645)
28. TYH medium (see Recipes)

Equipment

1. #5 forceps (DUMONT, model: 11252-20)
2. Appropriate CO_2 incubator (ASTEC, model: SCA-30DR) (for maintaining a 5% CO_2 atmosphere)
3. Appropriate fluorescent inverted microscope (*e.g.*, Olympus, model: IX71)
4. Appropriate stereo microscope (*e.g.*, Olympus, model: SZX16)
5. Egg-handling mouth pipette (DRUMMOND, model: 2-040-000), which has a joint with fire-polished glass tube of approximately 150-μm in diameter (DRUMMOND, model: 2-000-100) and a mouthpiece (MINATO MEDICAL CORPORATION, model: KA239-02), in accordance with instructions from a reputable textbook (*e.g.*, Behringer *et al.*, 2014) (Figure 1A)
6. Fine scissors (Natsume Seisakusho, model: B12-H)
7. Straight-blade Vannas scissors (Natsume Seisakusho, model: MB-54-1)
8. Suitable thermal plate (*e.g.*, TOKAI HIT, model: TPi-SZX2X) (it is advisable to use a thermal plate to maintain a temperature of 37°C on the stereo microscope in all experiments)

Software

1. OriginPro 2020b (LightStone)

Procedure

A. Preparation of oocytes
   1. Perform intraperitoneal injections of 7.5 IU eCG and 7.5 IU hCG at a 48-h interval to ≥ 8-week-old female mice of an appropriate strain, using a 1-ml, 26-gauge, 1/2-inch syringe [*e.g.*, four hybrid B6D2F1 female mice for an experiment when wild-type mice are used (approximately 150 oocytes are ovulated)].
   2. Sacrifice the mice 15-17 h after hCG injection.
   3. Dissect the oviducts in accordance with instructions from a reputable textbook, using fine scissors and #5 forceps (*e.g.*, Behringer *et al.*, 2014).
   4. Transfer the oviducts to a mineral oil-covered 35-mm plastic dish.
   5. Newly ovulated oocytes, surrounded by cumulus cells [cumulus-oocyte complex (COC)], are found in the ampulla of the oviduct.

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6. Place one oviduct beside a mineral oil-covered 50-µl drop of TYH medium in a 60-mm plastic dish (Figure 1B and 1C).
7. Use #5 forceps to grasp the oviduct, and supplementary forceps to create a tear in the oviduct close to where the oocytes are located (Figure 1C).

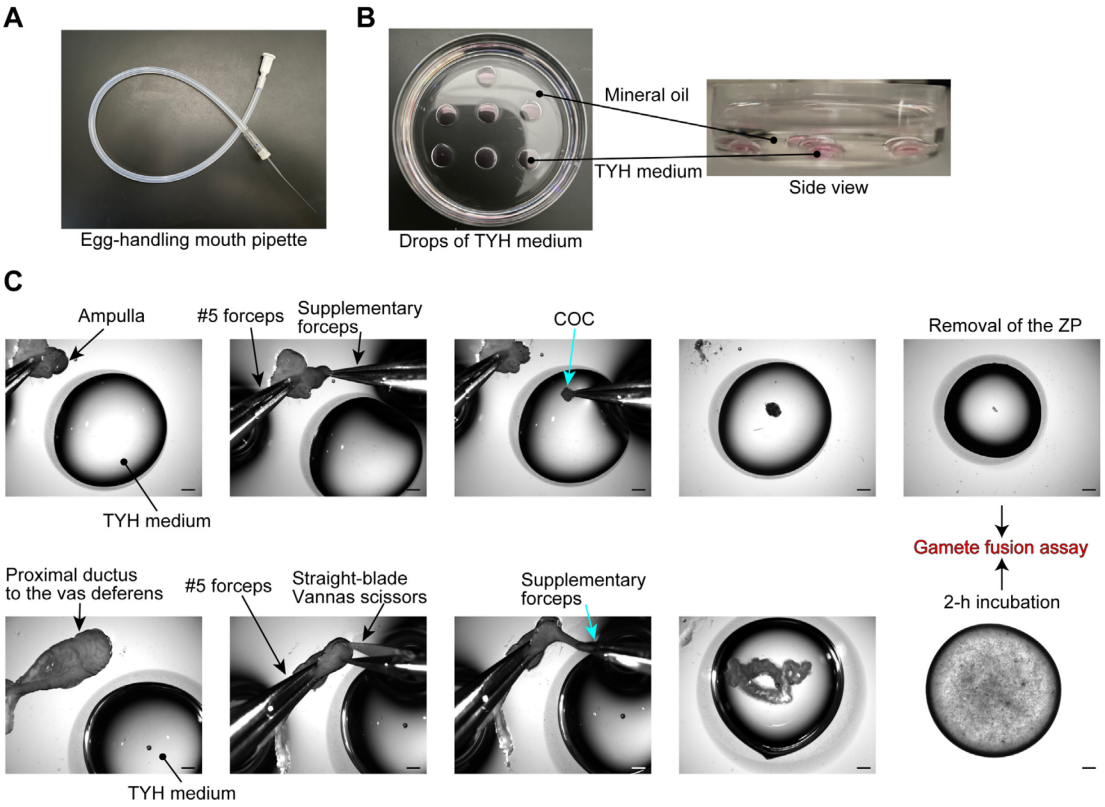

Figure 1. Preparation prior to gamete fusion assay.

A. Image of an egg-handling mouth pipette. B. Preparation of TYH drops. TYH medium is covered by pouring mineral oil after making TYH drops. C. Preparation of gametes. Oocyte preparation and spermatozoon preparation are shown in the top and bottom rows, respectively. These pictures correspond to Subheadings A and C, respectively. Scale bars: 1 mm.

B. Removal of the zona pellucida (Figure 2)

The gamete fusion assay is widely used for assessing the establishment of membrane fusion, for which most studies employ the Hoechst 33342 pre-load method. Please be careful during the ZP removal procedure prior to visualization of fused spermatozoa.

1. Acidic Tyrode's solution method
   a. Release the clutch of the COC into a 50-µl drop of TYH medium with 0.33 mg/ml hyaluronidase in a 60-mm plastic dish (Figure 1C).
   b. Allow a few COCs to incubate in a 50-µl drop of TYH medium with 0.33 mg/ml hyaluronidase at 37°C under 5% CO_2 in air, until the cumulus cells are completely removed (within 5 min).
   c. Wash the cumulus-free oocytes by pipetting in and out using an egg-handling mouth pipette

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(Figure 1A), and subsequently transfer the oocytes into fresh 50-µl drops of TYH medium (repeat washing at least three times).

d. Transfer the cumulus-free oocytes into a 50-µl drop of mineral oil-covered acidic Tyrode's solution prepared in a 60-mm plastic dish.
e. To remove the remnant medium, transfer the oocytes into a second 50-µl of acidic Tyrode's solution.
f. Repeatedly pipette the oocytes in and out until the ZP are dissolved under the stereoscopic microscope [it should occur within 30 s; otherwise, the survival rate of the oocytes will be significantly decreased (dead oocytes will be ruptured)].
g. Wash the ZP-free oocytes at least three times by transferring them into fresh new 50-µl drops of TYH medium, to remove the remnant acidic Tyrode's solution.
h. Incubate the ZP-free oocytes in a 50-µl drop of TYH medium for more than 1 h at 37°C under 5% CO_2 in air, to allow surface proteins to recover.

2. Collagenase method
   a. Release the clutch of the COC into a 50-µl drop of TYH medium with 1 mg/ml of collagenase in a 60-mm plastic dish.
   b. Incubate for 30 min at 37°C under 5% CO_2 in air.
   c. Carefully pick up the ZP-free oocytes with an egg-handling mouth pipette.
   d. Wash the ZP-free oocytes at least three times by transferring them into fresh new 50-µl drops of TYH medium.
   e. Maintain the ZP-free oocytes in TYH medium at 37°C under 5% CO_2 in air until use.

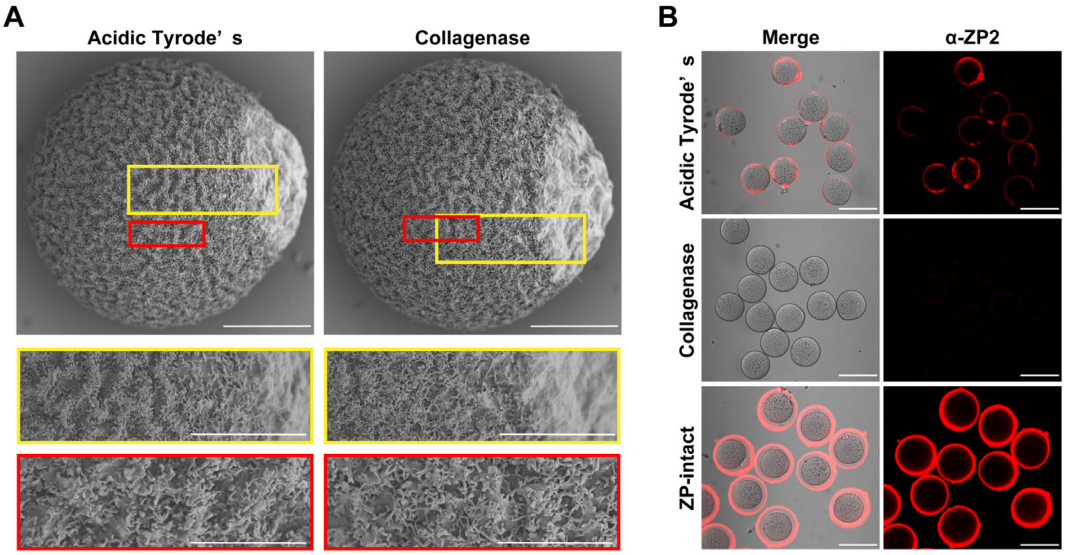

Figure 2. Comparison between acidic Tyrode's solution and collagenase-treated ZP-free oocytes.

A. High-resolution ultrastructure of the ZP-free oocytes imaged by a field emission scanning electron microscope (FE-SEM). There were no differences in the surface structure of oocytes obtained by the two methods. The yellow and red boxes correspond to each magnified image.

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Scale bars: 15 μm (top); 10 μm (middle); 5 μm (bottom). B. Visualization of a surface remnant of the dissolved ZP by monoclonal antibody (IE-3) against mouse ZP2, which is the major component of ZP. ZP-free oocytes prepared by the two methods were stained with 2 μg/ml IE-3. Subsequently, the antibody was detected using 5 μg/ml Alexa Fluor 647-labeled goat α-rat IgG (H+L) antibody. The images were taken with a confocal microscope. A remnant of the dissolved ZP was still found on the oocyte surface when the acidic Tyrode's solution method was used, whereas they were undetectable when the collagenase method was employed. Scale bars: 100 μm.

C. Collection of mature spermatozoa

1. Dissect the cauda epididymis from ≥ 12-week-old male mice of an appropriate strain, using fine scissors and #5 forceps, in accordance with instructions from a reputable textbook (*e.g.*, Behringer *et al.*, 2014) (*e.g.*, one hybrid B6D2F1 male mice for an experiment when wild-type mice are used).
2. Transfer the cauda epididymis to a mineral oil-covered 35-mm plastic dish.
3. Place the epididymis beside a 200-μl drop of TYH medium covered by mineral oil in a 60-mm plastic dish (Figure 1C).
4. Use #5 forceps to grasp the cauda epididymis, and make a cut proximally to the thick ductus of the vas deferens, where motile spermatozoa are stored, with straight-blade Vannas scissors (Figure 1C).
5. After squeezing the spermatozoa out from the cut end, hold a swarm of spermatozoa by sticking them to the tip of the supplementary forceps (Figure 1C).
6. Introduce the swarm of spermatozoa into a 200-μl drop of TYH medium (Figure 1C).
7. One hour after incubation, check sperm motility by observing the well-dispersed spermatozoa, and calculate the sperm concentration.
8. Cultivate the spermatozoa for an additional 1 h in the TYH medium at 37°C under 5% CO_2 in air, to induce capacitation and spontaneous acrosome reaction before insemination.

D. Visualization of fused spermatozoa

1. Prepare the ZP-free oocytes as described in Subheading B.
2. Introduce the ZP-free oocytes into a 50-μl drop of 1 μg/ml Hoechst 33342 [the alternative reagent is a 4′,6-diamidino-2-phenylindole (DAPI) (Kaji *et al.*, 2000)] TYH medium (up to 50 oocytes per spot) in a 60-mm plastic dish, and allow to stand for 10 min at 37°C under 5% CO_2 in air.
3. Transfer the oocytes into another fresh 50-μl drop of TYH medium covered with mineral oil.
4. Incubate dye-loaded oocytes for 15 min at 37°C under 5% CO_2 in air to release any excess dye.
5. Repeat Steps 3 and 4 three more times, and subject the oocytes to the gamete fusion assay.
6. Cultivate the spermatozoa as described in Subheading C to induce capacitation.

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7. Incubate dye-loaded oocytes with 2 × 10^5 spermatozoa/ml in a 100-µl drop of TYH medium for 30 min at 37°C under 5% CO_2 in air.
8. Transfer the sperm-bound eggs into a 50-µl drop of FHM medium containing 0.25% glutaraldehyde for slow fixation [fixation can be omitted at this stage for live imaging (Satouh *et al.*, 2012)].
9. Allow to stand for 5 min at room temperature.
10. Wash the sperm-bound eggs by transferring them into fresh drops of FHM medium three times without pipetting in and out.
11. Observe under a fluorescence microscope (20× or 40× objective lens, 405 nm excitation light). This procedure enables the staining of the nuclei of fused spermatozoa only, by transferring the dye into the spermatozoa after membrane fusion, whereas non-fused spermatozoa are undetectable. Chromosomes during meiosis metaphase II have the appearance of a cluster (Figure 3).
12. Manually change several focuses of the inverted microscope, and count the number of fused spermatozoa per oocyte.

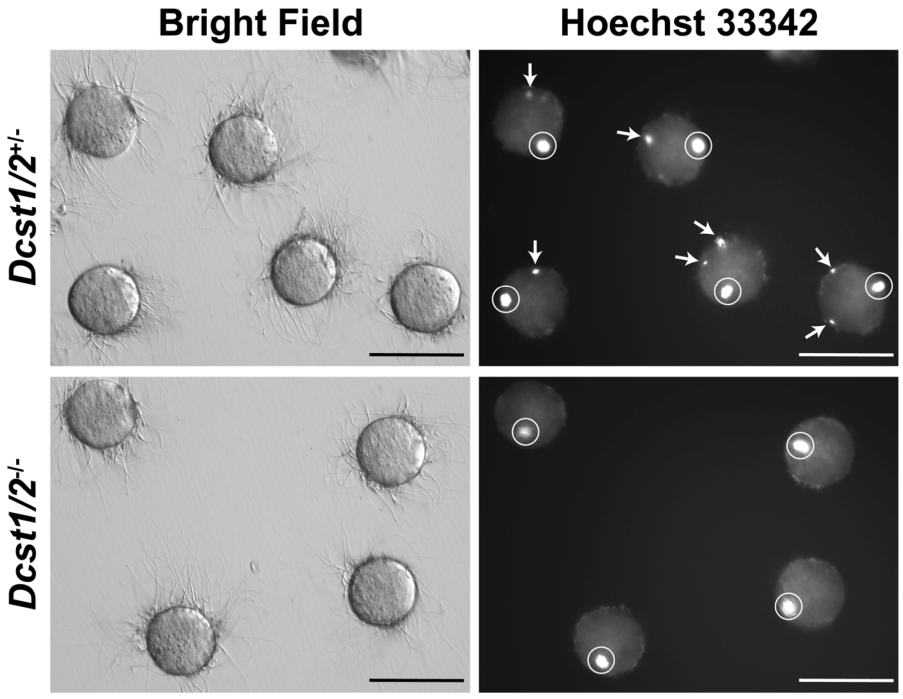

Figure 3. Example of gamete fusion assay by the fluorescent dye transfer method: *Dcst1/2* disrupted spermatozoa are unable to fuse with the oocytes.

Fused spermatozoa were stained with Hoechst 33342 pre-loaded on the oocytes. The arrows and circles show the fused spermatozoa and meiosis metaphase II chromosomes, respectively. A few *Dcst1/2*$^{+/-}$ spermatozoa were successfully fused with the oocytes, whereas *Dcst1/2*$^{-/-}$ spermatozoa fusion with the oocytes never occurred. Scale bars: 100 µm.

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Data analysis

Capture images with an appropriate fluorescent inverted microscope (*e.g.*, Olympus IX71) and analyze them using the built-in software. After counting the fused spermatozoa under the microscope, perform statistical analyses using OriginPro 2020b (LightStone).

Recipes

1. TYH medium
   6.976 g/L NaCl
   0.356 g/L KCl
   0.162 g/L KH_2PO_4
   1.0 g/L Glucose
   0.056 g/L Sodium pyruvate
   0.252 g/L $CaCl_2 \cdot 2H_2O$
   0.294 g/L $MgSO_4 \cdot 7H_2O$
   2.106 g/L $NaHCO_3$
   4 g/L AlbuMAX™ I Lipid-Rich BSA
   10 ml/L Penicillin-streptomycin
   15 ml/L Phenol red 0.04% (w/v)
   Adjust to 1 L with sterile, endotoxin free water
   After filtering through a 0.22-μm pore size polyethersulfone membrane, dispense TYH medium to the appropriate volume of polystyrene conical tubes, and store at -20°C until use. Equilibrate TYH medium with CO_2 before use.

Acknowledgments

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Competing interests

The author declares no financial competing interests.

Ethics

All animal studies were approved by the Animal Care and Use Committee of Fukushima Medical

University, Japan (approval number: 2020017) and performed under the guidelines and regulations of Fukushima Medical University.

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